IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

DOUGLAS N. ISHII

Serial No.: 08/571,802

Filed: February 17, 1998

For: METHOD FOR TREATING STROKE OR TRAUMATIC INJURY TO THE CENTRAL NERVOUS SYSTEM WITH IGF-I OR IGF-II

Group Art Unit: 1646

Examiner: M. Pak

Attorney Docket: CSUA019--1/WAA

DECLARATION OF DOUGLAS N. ISHII UNDER 37 C.F.R. § 1.132

I, Douglas N. Ishii, declare:

1. I am a professor at the Colorado State University College of Veterinary Medicine and Biomedical Sciences and have held that position since 1989. I have been studying and working with neurotropic factors since 1974.

2. I am the inventor of the subject matter that is claimed and for which a patent is sought on the invention titled "Method for Treating Stroke or Traumatic Injury to the Central Nervous System with IGF-I or IGF-II." I have reviewed and understand the contents of this application.

3. I understand that the specification is objected to and claims are rejected under 35 U.S.C. § 112, first paragraph, based on the enablement requirement. The following information indicates that the specification does enable the pending claims.

4. The Locus Ceruleus Model in Example III of the Specification

Example III of the specification, which was conducted by me or under my supervision, demonstrated that chemically-induced damage to the locus ceruleus can be ameliorated by parenteral administration of IGF-II. In that example, Sprague Dawley rats were inject with 6-hydroxydopamine to damage noradrenergic locus ceruleus cells in the pons of the brain. The location of the locus ceruleus cells and axons is depicted in Kandel et al., *Principles of Neural Science*, 3d ed. (1991) p. 694 (Ex. A). When these cells were damaged, the associated axons, which descend down the spinal cord and synapse on interneurons, were destroyed as manifested by a loss in the duration and amplitude of the hind limb reflex force. Lesioned rats that were parenterally administered IGF-II, however, were significantly spared from loss of hind limb reflex force. Example II of the specification also demonstrated that IGF-I effectively ameliorated impairment of the central nervous system by diabetes.

5. Additional Data for the Locus Ceruleus Model with IGF-I

The following experiment demonstrates that parenteral administration of IGF-I ameliorates chemically-induced damage to the locus ceruleus. This experiment was conducted in the same manner as Example III, except that IGF-I was used in place of IGF-II and the results were based on the measurements of groups of rat subjects. Sprague Dawley rats were treated with 6-hydroxydopamine to lesion the noradrenergic axons arising from the brainstem and descending down the spinal cord. Following lesioning, rats were implanted with subcutaneous pumps that released either vehicle or IGF-I for 1 week, and the hind limb withdrawal reflex force was measured after 3 weeks. Fig. 1 (Ex. B) shows the mean maximal force of hind-limb reflex in various groups of rats: NL, nonlesioned rats; L + Veh, lesioned rats treated with subcutaneous

pumps releasing vehicle; L + IGF-I, lesioned rats treated with subcutaneous pumps releasing IGF-I (4.8 ug/rat/day). The means + SEM (standard estimate of the mean) are shown for each group. *P < 0.009 (L + IGF-I) vs. (L + Veh); **P < 0.0002 (NL) vs. (L + Veh). These data show that the mean force of hind-limb withdrawal was significantly reduced in lesioned versus nonlesioned rats. Moreover, this force was significantly preserved in IGF-I treated versus vehicle treated lesioned rats. Therefore, subcutaneous treatment with human IGF-I acted across the blood brain barrier to preserve function in a central nervous system lesioned mammal.

The spinal cords from these rats were subsequently sectioned to determine whether IGF treatment could preserve the noradrenergic circuitry in the lumbar region of the spinal cord containing the motoneurons involved in the hind limb reflex. Dopamine-ß-hydroxylase (DBH) is an enzyme in the pathway producing noradrenaline and can be used to identify the noradrenergnic axons in the spinal cord. Such axons are beaded with varicosities containing neurotransmitters. An anti-DBH antibody was tested and found to selectively stain the noradrenergic neurons in the locus ceruleus but not other cells in the surrounding area. Using this antibody, the noradrenergic axons and vesicles were detected in the lumbar region of the spinal cord of nonlesioned rats.

A total of six sections (30 μm sections taken every 5 mm) from the lumbar region of each rat of each treatment group (N - 7 or 8 rats per group) were subjected to morphometric analysis using the Metamorph system (Universal Imaging Corp., West Chester, PA). Fig 2 (Ex. C) shows a region of the spinal cord stained with antibody showing noradrenergic varicosities arranged like beads on axons. The boxed region is a standard area selected for analysis. Fig. 2B shows the varicosities selected in the standard area for computer analysis. The mean number of varicosities

per unit spinal cord area was calculated for each rat group. Figure 2C shows means + SEM for each group. *P < 0.01 for (L + IGF-I) vs. (L + Veh); **P < 0.0002, (L + Veh) vs. NL. These data show that the mean number of adrenergic varicosities was significantly reduced in lesioned rats. Moreover, the mean number of adrenergic varicosities was significantly preserved in IGF-I treated versus vehicle-treated lesioned rats. Thus, subcutaneously administered IGF can act across the blood brain barrier to prevent loss of spinal cord circuitry as well as preserve function in a mammal with a locus ceruleus lesion.

6. Scientific Publications Acknowledge Locus Ceruleus Damage in Neurodegenerative Trauma and Disorders

I have reviewed the scientific literature relating to locus ceruleus damage in neurodegenerative trauma and disorders. The locus ceruleus is involved in many diseases and disorders, which I believe are susceptible to treatment by the invention based on the teachings of the specification and the above-mentioned locus ceruleus model in rat. The following conditions are recognized as involving disease or damage to the locus ceruleus:

(a)	Alzheimer's Disease	Mann et al., *Clinical Neuropathology*, 2:1-7 (1983) (Ex. D); Chan-Palay et al., *J. Comparative Neurology*, 287:373-392 (1989)(Ex. E);
(b)	Parkinson's Disease	Mann et al., *Clinical Neuropathology*, 2:1-7 (1983); Chan-Palay et al., *J. Comparative Neurology*, 287:373-392 (1989);
(c)	Dementia pugilistica	Mann et al., *Clinical Neuropathology*, 2:1-7 (1983);
(d)	Progressive supernuclear palsy	Mann et al., *Clinical Neuropathology*, 2:1-7 (1983);
(e)	Huntington's Disease	Zweig et al., *Annals of Neurology* 24:233-242 (1988) (Ex. F);
(f)	Pick's Disease	Arima et al., *Acta Neuropathologica* 79:629-33, (1990) (Ex. G);

(g)	Major depression	Chan-Palay et al., *J. Comparative Neurology*, 287:373-392 (1989);
(h)	Suicide	Arango et al., *Biol. Psychiatry* 39:112-120 (1996) (Ex. H);
(i)	Schizophrenia	Chan-Palay et al., *J. Comparative Neurology*, 287:373-392 (1989);
(j)	Down's Syndrome	Mann et al., *Clinical Neuropathology*, 2:1-7 (1983);
(k)	Shy-Drager Syndrome	Sima et al., *Clinical Neuropathology* 6:49-54 (1987) (Ex. I);
(l)	Rett Syndrome	Nomura et al., *Brain & Development* 7:334-341 (1985) (Ex. J); and
(m)	Olivopontocerebellar atrophy	Grijalba et al., *J. Neural Transmission* 96:135-42 (1994) (Ex. K).

7. Experiments Further Demonstrating Parenterally-Administered IGFs Crossing the Blood-Brain Barrier

An osmotic minipump containing human IGF-I was implanted subcutaneously in the mid-back region of a rat to continuously deliver human IGF-I. Cerebrospinal fluid (CSF) was removed from the rats prior to treatment (zero time) and after 0.5, 1, 2 and 3 days of human IGF-I infusion. The CSF was subjected to acid-ethanol extraction to dissociate IGF from IGF binding proteins. The human IGF-I in rat CSF was measured using a very sensitive ELISA assay that detected human IGF-I but not human IGF-II, insulin, rat IGF-I, rat IGF-II or rat insulin. The anti-IGF antibody was coupled to horse radish peroxidase, and tetramethylbenzidine substrate was used to produce a colored product that permitted detection of immunoreactive human IGF-I (ihIGF-I) at O.D. 450 nm.

Fig. 3 (Ex. L) shows that human IGF-I was measured in rat CSF after 0.5, 1, 2 and 3 days. It was not detected at 0 days, and the lack of signal confirmed that the assay did not detect rat IGF-I, IGF-II or insulin in rat CSF. These data show that human IGF-I administered subcutaneously can cross the blood brain barrier and enter the CSF.

In the second example, human ^{125}I-IGF-I was injected subcutaneously in a rat. After 2 hours, CSF (100 µl) was carefully withdrawn with a syringe mounted on a micromanipulator after surgical exposure of the atlanto-occipital membrane. Serum was also withdrawn. The treated rat CSF and serum, and other test samples, were subjected to polycrylamide gel electrophoresis in sodium dodecylsulfate-containing (SDS PAGE) gels. This gel separates proteins on the basis of size. Fig. 4 (Ex. M) shows a silver stain of the gel (upper panel) and autoradiogram of the same gel (lower panel). The lanes contained the following samples (from left to right): protein molecular weight markers; BSA buffer used to block potential nonspecific adsorption of radioactive IGF to assay tubes; standard ^{125}I-IGF-I plus CSF from untreated rat (shows normal migration position of standard ^{125}I-IGF-I is not influenced by mixing with CSF); standard ^{125}I-IGF-I plus BSA (shows normal migration position of ^{125}I-IGF-I); radioactive CSF from treated rat (shows ^{125}I-IGF injected subcutaneously crosses the BBB and has size indistinguishable from standard intact ^{125}I-IGF-I); standard nonradioactive human IGF-I (shows position of migration on silver-stained gel at 7.5 kDa size); and radioactive plasma from treated rat (shows ^{125}I-IGF-I administered subcutaneously accumulates in plasma to permit crossing the BBB).

Taken together, these data show that human IGF administered subcutaneously *in vivo* in a mammal can accumulate in serum and cross the blood brain barrier as intact IGF.

8. I further declare that all of these statements based on my knowledge are true and all statements made on information and belief are believed to be true. These statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

11-12-98
Date

Dr. Douglas N. Ishii